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Support for the Amendment

Amendments have been made to more clearly recite the invention the applicants intent to claim. Support for new claims 48 and 49 is provided, for example, by page 22, lines 2-7 and Figures 4 and 7C, where such cofactor binding sites are disclosed. Support for new claim 50 is provided, for example, by Fig. 7C. New claims 51 and 52 specify that the nucleic acid decreases the activity of a mutant or polymorphic methionine synthase reductase polypeptide in a subject, as disclosed on page 10, lines 5-12, and page 22, lines 14-18. New claim 53 specifies that the nucleic acid has a naturally-occurring mammalian methionine synthase reductase mutation (see, for example page 5, lines 17-18 and page 6, lines 1-11).

The Sequence Listing was amended to include the FMN, FAD, and NADPH binding sites of human methionine synthase reductase, as disclosed in Fig. 4 (SEQ ID NOs: 52-61) and as recited in new claims 48 and 49.

No new matter is added by these amendments.

Claim Objection

Claims 36-38 and 41-43 were objected because of improper sequence identifiers. As suggested by the Examiner, applicants have amended these claims to use the phrase "SEQ ID NO" in the sequence identifiers. Accordingly, this objection may now be withdrawn.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 3 and 47 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failure to distinctly claim the subject matter which applicants regard as the invention. As suggested by the Examiner, applicants have added the word "respectively" to claim 3 to clarify that each of the recited nucleic acid sequences encodes one of the

recited amino acid sequences. Accordingly, this aspect of the rejection may be withdrawn.

The Examiner states that claim 47 is indefinite for use of the phrase "consensus binding site for one or more cofactors." Applicants respectfully assert that one skilled in the art would readily recognize a consensus binding site for FAD, FMN, and/or NADPH cofactors. In particular, numerous proteins have been characterized as FAD, FMN, and/or NADPH binding proteins based on the presence of consensus binding sites for these cofactors. Applicants enclose herewith in Appendix A an exemplary reference published prior to applicants' earliest priority date (January 16, 1998) that teaches the characteristics of these binding sites in members of the CPR family which includes methionine synthase reductase (Wang *et al.*, Proc. Natl. Acad. Sci. USA 94:8411-8416, 1997). Enclosed Appendix B contains the sequence alignment of CPR family members from this reference (Fig. 6) to which applicants have added the corresponding sequence of human methionine synthase reductase and boxed the residues that are identical among the listed CPR family members. As noted in the attached Declaration of Dr. Roy Gravel, the FMN, FAD, and NADPH binding regions are highly conserved between human methionine synthase reductase and other CPR family members. Thus, a skilled artisan can readily identify the cofactor binding sites in human and other mammalian methionine synthase reductases. As consensus binding sites for FAD, FMN, and/or NADPH cofactors are well known to those of skill in the art, this indefiniteness rejection should be withdrawn.

Applicants further note that new claims 48-50 specify particular sequences for FAD, FMN, and/or NADPH binding sites (as disclosed, for example, page 22, lines 2-7 and Figures 4 and 7C). In particular, claims 48 and 49 specify that the sequence of the binding site is at least 70% or 100% identical, respectively, to one of the FAD, FMN, and/or NADPH binding sites in human methionine synthase reductase (page 22, lines 2-7 and Fig. 4). New claim 50 specifies that the binding site is identical to one of the

NADPH binding sites found in an FNR family member (Fig. 7C). Thus, new claims 48-50 also clearly recite subject matter of the originally disclosed invention.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1, 2, 4, 5, and 35-47 were rejected under 35 U.S.C. § 112, first paragraph, for failure of the specification to convey possession of the scope of the claimed invention. The Examiner states that specification fails to disclose a sufficient number of mammalian methionine synthase reductase nucleic acids and fails to describe structural features of mammalian methionine synthase reductase nucleic acids. Applicants respectfully disagree.

Rejection for failure to describe a sufficient number of mammalian methionine synthase reductase nucleic acids sequences

The specification discloses the polynucleotide sequences of several full-length mammalian methionine synthase reductase nucleic acid, including SEQ ID NOs: 1, 41, 43, 45, and 47 (page 6, lines 1-10). In addition to structurally characterizing these mammalian methionine synthase reductase nucleic acids by their exact polynucleotide specification, the applicants' specification teaches that mammalian methionine synthase reductase nucleic acids are structurally characterized by the regions encoding the consensus binding sites for FAD, FMN, and NADPH (page 28, lines 10-13; page 29, lines 19-25; and Figures 2, 4, and 7). Additionally, mammalian methionine synthase reductase nucleic acids are characterized by the specific ability of the encoded protein to generate cob(III)alamin-CH_3 from the reductive methylation of cob(II)alamin or to increase methionine synthase activity by maintaining the cobalamin cofactor of methionine synthase in an active state (page 16, lines 2-5; page 34; and page 35). Applicants respectfully assert that these distinguishing characteristics sufficiently characterize mammalian methionine synthase reductase nucleic acids such that one skilled in the art

could readily identify a mammalian methionine synthase reductase nucleic acid.

In particular, mammalian methionine synthase reductase nucleic acids can be readily identified based on their sequence identity to the polynucleotide sequences of SEQ ID NOs: 1, 41, 43, 45, or 47 using standard sequence comparison software such as the Sequence Analysis Software Package of the Genetics Computer Group disclosed on page 18, lines 22-25 of the specification. In particular, the asterisks in Fig. 4 denote amino acids that are identical between human methionine synthase reductase and the distantly related *C. elegans* putative methionine synthase reductase, and the filled circles in Fig. 4 denote conservative substitutions or identical amino acids between human and *C. elegans* methionine synthase reductases. Given the surprising degree of identity between the mammalian (*e.g.*, human) methionine synthase reductase that was discovered by the applicants and non-mammalian methionine synthase reductase (*e.g.*, *C. elegans* putative methionine synthase reductase), one skilled in the art would appreciate that these residues will also almost certainly be conserved in other mammalian synthase reductase genes. Fig. 4 also indicates the conserved regions in human and *C. elegans* methionine synthase reductases that are involved in FAD, FMN, and/or NADPH binding. Given the numerous conserved regions between human and *C. elegans* methionine synthase reductases, there is a great level of predictability inherent in the practice of the invention. In fact, one skilled in the art would expect a higher degree of conservation among these regions between the human and other mammalian methionine synthase reductase genes than between the human and *C. elegans* genes.

Furthermore, one skilled in the art could readily use standard assays such as those disclosed in the specification to measure the enzymatic activity of the encoded mammalian methionine synthase reductase protein. Applicants note that claims 45 and 46 have the additional limitation of requiring that the encoded mammalian methionine synthase reductase polypeptide has at least 20-30% or at least 55-75%, respectively, of the ability to catalyze the reductive methylation of methionine synthase-cob(II)alamin to

generate methionine synthase-cob(III)alamin-CH3 as the methionine synthase reductase polypeptide of SEQ ID NO: 2.

As multiple distinguishing characteristics that are shared by mammalian methionine synthase reductase nucleic acids are disclosed in applicants' specification, this aspect of the rejection should be withdrawn.

Rejection for failure to describe a multiple nucleic acids that hybridize to a mammalian methionine synthase reductase nucleic acid

In response to the Examiner's assertion that the specification does not sufficiently describe characteristics of nucleic acids that hybridize at high stringency to the methionine synthase reductase nucleic acids of SEQ ID NOs: 1 or 41, applicants note that the specification structurally characterizes mammalian synthase reductase nucleic acids of SEQ ID NOs: 43, 45, and 47, which hybridize to SEQ ID NOs: 1 and 41, by providing their polynucleotide sequences. The specification also provides guidance for nucleic acids that contain regions from SEQ ID NOs: 43, 45, or 47 which would also hybridize to SEQ ID NOs: 1 and 41. For example, as stated on page 17, line 15 through page 18, line 1 of the specification:

> Probes or primers specific for methionine synthase reductase nucleic acid preferably will have at least 35% sequence identity, more preferably at least 45-55% sequence identity, still more preferably at least 60-75% sequence identity, still more preferably at least 80-90% sequence identity, and most preferably 100% sequence identity. Probes may be detectably-labelled, either radioactively, or non-radioactively, by methods well-known to those skilled in the art. Probes are used for methods involving nucleic acid hybridization, such as: nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, Northern hybridization, in situ hybridization, electrophoretic mobility shift assay (EMSA).

In addition, Table 1 of the specification discloses the polynucleotide sequence of twenty primers that were using in the cloning, mapping, and mutation detection of human

methionine synthase reductase (page 48). These nucleic acids are characterized by their ability to hybridize to SEQ ID NOs: 1 and 41, using standard methods such as those disclosed on page 16, lines 19-23 of the specification:

> By "high stringency conditions" is meant hybridization in 2X SSC at 40°C with a DNA probe length of at least 40 nucleotides. For other definitions of high stringency conditions, see Ausubel et al., Current Protocols in Molecular Biology, pp. 6.3.1-6.3.6, John Wiley & Sons, New York, NY, 1998, hereby incorporated by reference.

Antisense nucleic acids, which also hybridize to methionine synthase reductase nucleic acids, are further characterized by their sequence complementarity to a methionine synthase reductase nucleic acid and by their ability to decrease methionine synthase reductase activity (page 22 and 24). For example, new claims 51 and 52 further limit claims 4 and 41 to nucleic acids that lead to a decrease in the activity of a mutant or polymorphic methionine synthase reductase polypeptide in a subject who is administered a nucleic acid. In particular, the applicants' specification teaches:

> By "antisense" as used herein in reference to nucleic acids, is meant a nucleic acid sequence that is complementary to the coding strand of a gene, preferably, a methionine synthase reductase gene. An antisense nucleic acid is capable of preferentially decreasing the activity of a mutant methionine synthase reductase polypeptide encoded by a mutant methionine synthase reductase gene.

Page 22, lines 14-18.

The specification further describes numerous assays that can be readily used by one skilled in the art to characterize the ability of an antisense nucleic acid to reduce methionine synthase reductase activity.

> By "a decrease" is meant a lowering in the level of biological activity, as measured by inhibition of: a) the formation of enzymatically active methionine synthase-cob(III)alamin-CH3 or methionine as a result of methionine synthase reductase activity; b) protein, as measured by ELISA; c) reporter gene activity, as measured by reporter gene assay, for example, lacZ/β-galactosidase, green fluorescent protein, luciferase, etc.; or d) mRNA, as measured by PCR relative to an internal control, for example, a "housekeeping" gene product such as β-actin or

> glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In all cases, the decrease is preferably by at least 10% more preferably by at least 25%, still more preferably by at least 50%, and even more preferably by at least 70%.

Page 24, lines 9-18.

Applicants note that claims 4, 5, 35, and 41-44 specify that the nucleic acid has a region that is complementary to a naturally-occurring mammalian methionine synthase reductase mutation or polymorphism. The specification discloses numerous polymorphic methionine synthase reductase nucleic acids in which (i) a guanine is replaced by adenine at nucleotide position 66 (SEQ ID NO: 41), (ii) a guanine is replaced by adenine at nucleotide position 110 (SEQ ID NO: 43), (iii) nucleotides 1675-1678 are deleted (SEQ ID NO: 47), or (iv) nucleotides 1726-1728 are deleted (SEQ ID NO: 45) (page 6, lines 1-10). The specification also describes other exemplary mutations on page 14, 15, 25, and 26. For example, page 15, lines 7-22 of the specification states:

> Such mutations may be naturally occurring, or artificially induced. They may be, without limitation, transition, transversion, insertion, deletion, frameshift, or missense mutations. A mutant methionine synthase reductase protein may have one or more mutations, and such mutations may affect different aspects of methionine synthase reductase biological activity (protein function), to various degrees. Alternatively, a methionine synthase reductase mutation may indirectly affect methionine synthase reductase biological activity by influencing, for example, the transcriptional activity of a gene encoding methionine synthase reductase, or the stability of methionine synthase reductase mRNA. For example, a mutant methionine synthase reductase gene may be a gene that expresses a mutant methionine synthase reductase protein or may be a gene which alters the level of methionine synthase reductase protein in a manner sufficient to confer a disease phenotype in at least some genetic and/or environmental backgrounds. The presence of polymorphic or mutant methionine synthase reductase may be determined by detecting polymorphic or mutant methionine synthase reductase nucleic acid or polypeptide, using methods that are known in the art.

To generate a nucleic acid that has a region that is complementary to a naturally-occurring mammalian methionine synthase reductase mutation or polymorphism, a skilled artisan can use a partial or full length nucleic acid that is complementary to a mutant or

polymorphic mammalian methionine synthase reductase nucleic acid, such as SEQ ID NOs: 41, 43, 45, or 47, or any other polymorphic mammalian methionine synthase reductase nucleic acid identified using the methods of the present invention. As multiple characteristics of nucleic acids that hybridize to mammalian methionine synthase reductase are taught in applicants' specification, this rejection should be withdrawn.

Claims 1, 2, 4, 5, 35, 36, 39-41, and 44-47 were rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. The Examiner acknowledges that the specification is enabling for a methionine synthase reductase nucleic acids of SEQ ID NOs: 1, 41, 43, 45, and 47. However, the Examiner asserts that the specification does not enable any mammalian or any human methionine synthase reductase nucleic acid, any degenerate variant of SEQ ID NOs: 1 or 41, or any nucleic acid that hybridizes at high stringency to a sequence within SEQ ID NOs: 1 or 41. In particular, the Examiner states that it is unpredictable what changes in a methionine synthase reductase nucleic acid maintain the activity of the encoded protein.

Applicants respectfully assert that the present claims are fully enabled by the specification. In response to the Examiner's argument that the specification has not provided sufficient guidance for the generation or isolation of any nucleic acid encoding a mammalian methionine synthase reductase polypeptide, applicants note that their disclosure of multiple human methionine synthase reductase nucleic acids enables one skilled in the art to determine the sequence of any other human methionine synthase reductase nucleic acid. For example, the rapid techniques for amplifying and sequencing a methionine synthase reductase nucleic acid, or a segment thereof, using the suggested oligonucleotide primers in Table 1 or any other primer allow a skilled artisan to determine the polynucleotide sequence of methionine synthase reductase from a patient sample, such as fibroblast cells (pages 17 and 42).

Given the human methionine synthase reductase nucleic acid sequences provided

by the applicants, one skilled in the art of molecular biology can also readily design PCR primers or probes to clone a representative number of mammalian methionine synthase reductase genes. As noted above, probes designed based upon binding sites for FAD, FMN, and NADPH, or any other probe (such as a partial or full length human methionine synthase reductase nucleic acid) may be used in routine methods to screen any mammalian nucleic acid library to identify additional mammalian methionine synthase reductase nucleic acids.

Additionally, other mammalian methionine synthase reductase nucleic acids can be readily generated using standard molecular biology techniques to mutate the polynucleotide sequences of SEQ ID NOs: 1, 41, 43, 45, or 47, or the polynucleotide sequences of any other mammalian methionine synthase reductase nucleic acids isolated using the methods of the invention. The activity of the encoded polypeptides can be rapidly tested by determining their enzymatic ability to catalyze the reductive methylation of methionine synthase-cob(II)alamin. Applicants note that claims 45 and 46, which require the nucleic acids to encode a polypeptide having at least 20-30% of the biological activity of the methionine synthase reductase polypeptide of SEQ ID NO: 2, clearly recite nucleic acids having substantial utility. Applicants further note that claims 47-50, which recite nucleic acids encoding a mammalian methionine synthase reductase polypeptide that has a consensus binding site for FAD, FMN, and/or NADPH, are directed to nucleic acids that maintain the ability to bind a cofactor.

In response to the Examiner's assertion that producing functional fragments or variants requires the determination of which fragments or variants retain enzymatic activity, applicants note that the claimed nucleic acids have numerous uses that do not require the encoded polypeptide to have enzymatic activity.

For example, the claimed nucleic acids are useful in diagnostic methods to detect mutations or polymorphisms associated with altered risk for a disease (see, for example, pages 8-11). In particular, the nucleic acids may be used in standard kits for detecting the

presence or absence of any base pair mutation, insertion, or deletion in a mammalian methionine synthase reductase nucleic acid (pages 25 and 26). As disclosed on page 9, lines 9-18, of the specification, the nucleic acids can be used as PCR primers to amplify and sequence a region of a methionine synthase reductase gene. The nucleic acids can also be used as probes for hybridization to mutant methionine synthase reductase genes (page 17). As disclosed by the applicants, these mutations may be associated with neural tube defects, cancer, or cardiovascular disease (pages 49-57). Subjects identified using these methods as having an increased risk for a disease may be administered a therapeutic agent to prevent or delay the onset of the disease. Applicants note that all of these diagnostic methods are dependent on properties of the claimed nucleic acids, instead of properties of the encoded polypeptides, and thus do not require the nucleic acids to encode functional polypeptides.

Moreover, the claimed nucleic acids may be used as therapeutic antisense nucleic acids to decrease the activity of a polypeptide encoded by a mutant methionine synthase reductase gene in a patient (page 22, lines 14-18). Again, the use of the claimed nucleic acids as antisense nucleic acids does not require the nucleic acids to encode functional polypeptides.

Given the various substantial clinical uses for these nucleic acids, it would be unfair to limit the present claims to nucleic acids that encode an active methionine synthase reductase protein.

As the applicants' disclosure of several human methionine synthase reductase nucleic acids enables the isolation or generation of additional mammalian methionine synthase reductase nucleic acids with a variety of disclosed uses, this enablement rejection should be withdrawn.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is requested. A marked-up version indicating the amendments made to the claims, as required by 37 C.F.R. § 1.121(c)(1)(ii), is enclosed.

Enclosed is a petition to extend the period for replying for three months, to and including May 16, 2002. Also enclosed is a check for the excess claims.

If there are any charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: May 16, 2002

Vicki L. Healy

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F:\50004\50004.003003 Reply to Office Action mailed 11.16.01 as filed.doc

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I hereby certify under 37 C.F.R. § 1.8(a) that this correspondence is being deposited with the United States Postal Service as **first class mail** with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Roy A. Gravel et al.	Art Unit:	1652
Serial No.:	09/371,347	Examiner:	D. Steadman
Filed:	August 10, 1999	Customer No:	21559
Title:	HUMAN METHIONINE SYNTHASE REDUCTASE: CLONING, AND METHODS FOR EVALUATING RISK OF NEURAL TUBE DEFECTS, CARDIOVASCULAR DISEASE, AND CANCER		

Assistant Commissioner for Patents
Washington, D.C. 20231

Version with Markings to Show Changes Made

Marked-up versions of claims 3, 36-38, and 41-43 and new claims 48-53 are presented below.

3. (Amended) The nucleic acid of claim 1, wherein said nucleic acid has the sequence of SEQ ID NO: 1, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, or SEQ ID NO: 47, or degenerate variants thereof, and wherein said nucleic acid encodes the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, or SEQ ID NO: 48, respectively.

36. (Amended) A substantially pure nucleic acid having a polynucleotide sequence that has at least 50% sequence identity to SEQ ID NO [No.]: 1 over the entire length of SEQ ID NO [No.]: 1.

37. (Amended) The nucleic acid of claim 36, having a polynucleotide sequence that has at least 85% sequence identity to SEQ ID NO [No.]: 1 over the entire length of SEQ ID NO [No.]: 1.

38. (Amended) The nucleic acid of claim 37, having a polynucleotide sequence that has at least 95% sequence identity to SEQ ID NO [No.]: 1 over the entire length of SEQ ID NO [No.]: 1.

41. (Amended) A substantially pure nucleic acid having a polynucleotide sequence that has at least 50% sequence identity to the corresponding region of SEQ ID NO [No.]: 1, wherein said nucleic acid comprises a naturally-occurring mammalian methionine synthase reductase mutation or polymorphism.

42. (Amended) The nucleic acid of claim 41, having a polynucleotide sequence that has at least 85% sequence identity to the corresponding region of SEQ ID NO [No.]: 1.

43. (Amended) The nucleic acid of claim 42, having a polynucleotide sequence that has at least 95% sequence identity to the corresponding region of SEQ ID NO [No.]: 1.

48. (New) The nucleic acid of claim 47, wherein said binding site comprises a sequence that is at least 70% identical to one of the following sequences:

(a) FLLLYATQQGQAKAIAEEMC (SEQ ID NO: 52),

(b) VVVVSTTGTGDPPDTARKFVKEI (SEQ ID NO: 53),

(c) AHLRYGLLGLGDSEYTYFCNGGKIIDKRL (SEQ ID NO: 54),

(d) LQPRPYSCASSSLFHPGKL (SEQ ID NO: 55),

(e) FVFNIVEFLSTATT (SEQ ID NO: 56),

(f) LRKGVCTGWLALLVASV (SEQ ID NO: 57),

(g) IPIIMVGPGTGIAPFIGFLQHR (SEQ ID NO: 58),

(h) SFSRDA (SEQ ID NO: 59),

(i) APAKYVQDNIQLHGQQVARILLQENGHIYVCGDAKNMAKDV (SEQ ID NO: 60), or

(j) KRYLQDIWS (SEQ ID NO: 61).

49. (New) The nucleic acid of claim 48, wherein said binding site comprises a sequence that is identical to one of the following sequences:

(a) FLLLYATQQGQAKAIAEEMC (SEQ ID NO: 52),

(b) VVVVSTTGTGDPPDTARKFVKEI (SEQ ID NO: 53),

(c) AHLRYGLLGLGDSEYTYFCNGGKIIDKRL (SEQ ID NO: 54),

(d) LQPRPYSCASSSLFHPGKL (SEQ ID NO: 55),

(e) FVFNIVEFLSTATT (SEQ ID NO: 56),

(f) LRKGVCTGWLALLVASV (SEQ ID NO: 57),

(g) IPIIMVGPGTGIAPFIGFLQHR (SEQ ID NO: 58),

(h) SFSRDA (SEQ ID NO: 59),

(i) APAKYVQDNIQLHGQQVARILLQENGHIYVCGDAKNMAKDV (SEQ ID NO: 60), or

(j) KRYLQDIWS (SEQ ID NO: 61).

50. (New) The nucleic acid of claim 47, wherein said binding site comprises any one of SEQ ID NOs: 25-40.

51. (New) The nucleic acid of claim 4 or 41, wherein the administration of said nucleic acid to a subject leads to a decrease in the activity of a mutant or polymorphic methionine synthase reductase polypeptide in said subject.

52. (New) The nucleic acid of claim 51, wherein the administration of said nucleic leads to a decrease in the activity of said mutant methionine synthase reductase polypeptide.

53. (New) The nucleic acid of claim 4 or 41, wherein said nucleic acid comprises said naturally-occurring mammalian methionine synthase reductase mutation.

\\Clark-w2k1\documents\50004\50004.003003 Reply to OA 11.16.01 marked up claims.doc

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Roy A. Gravel et al.	Art Unit:	1652
Serial No.:	09/371,347	Examiner:	D. Steadman
Filed:	August 10, 1999	Customer No.:	21559
Title:	HUMAN METHIONINE SYNTHASE REDUCTASE: CLONING, AND METHODS FOR EVALUATING RISK OF NEURAL TUBE DEFECTS, CARDIOVASCULAR DISEASE, AND CANCER		

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132 OF ROY A. GRAVEL, PH.D.

1. I am an inventor for the above-referenced patent application.

2. I have read the Office Action mailed November 16, 2001 in connection with the above-referenced patent application. I am a Professor and Killam Memorial Chair of the Department of Biochemistry & Molecular Biology at the University of Calgary.

3. One skilled in the art can readily determine the binding sites for FAD, FMN, and/or NADPH cofactors in a protein, such as mammalian methionine synthase, based on known binding sites for FAD, FMN, and/or NADPH cofactors in a protein from the same enzyme family.

In particular, Wang *et al.* (Proc. Natl. Acad. Sci. USA 94:8411-8416, 1997,

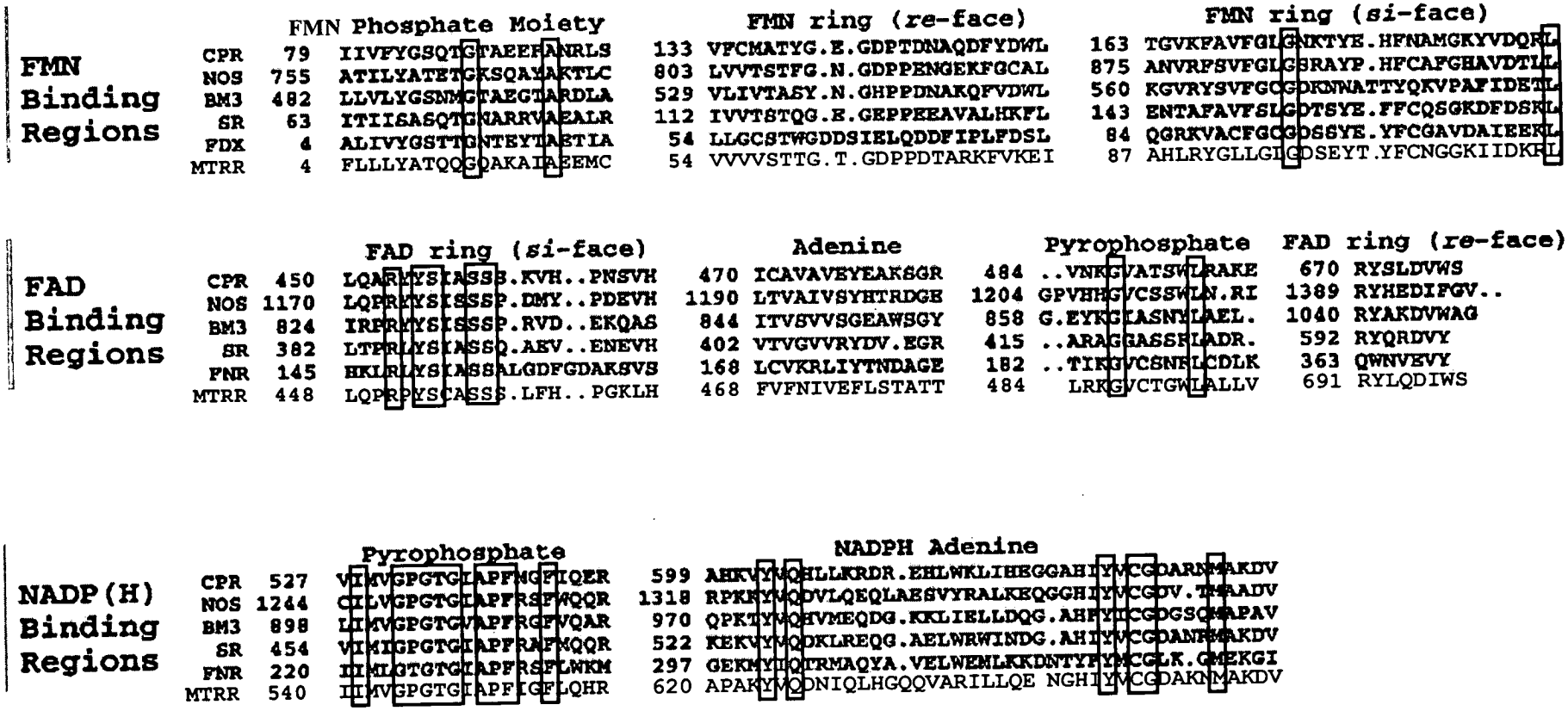
enclosed as Appendix A) report the three-dimensional structure of the FAD, FMN, and NADPH binding sites in microsomal NADPH-cytochrome p450 reductase (CPR). Fig. 6 of this reference also discloses the sequence alignment of the FAD, FMN, and NADPH binding sites of CPR with the corresponding regions in four other CPR family members. I have added the corresponding sequence of human methionine synthase reductase to this figure and boxed the residues that are identical among the listed CPR family members (Appendix B). Appendix B illustrates that the FMN, FAD, and NADPH binding regions are highly conserved between human methionine synthase reductase and other CPR family members. Thus, a skilled artisan can readily identify the cofactor binding sites in human and other mammalian methionine synthase reductases.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: May 9, 2002

Roy A. Gravel, Ph.D.

Appendix B



Alignment of ligand binding site sequences for FMN, FAD and NADPH containing enzymes. The figure is from Wang et al. (1997), below, with the addition of the corresponding sequence for methionine synthase reductase (MTRR in figure). The boxed areas of identity for all enzymes shown. CPR – rat cytochrome P450 reductase, NOS – rat neuronal nitric oxide synthase, BM3 – cytochrome P450 Bacillus megaterium 3, SR – E. coli sulfite reductase, FDX – D. vulgaris flavodoxin, FNR – Spinacia oleracea ferredoxin-NADP$^+$ reductase. Reference: Wang M, Roberts DL, Paschke R, Shea TM, Masters BS, Kim JJ. (1997) Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. Proc Natl Acad Sci U S A. 94:8411-8416.

Appendix A

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 8411–8416, August 1997
Biochemistry

Three-dimensional structure of NADPH–cytochrome P450 reductase: Prototype for FMN- and FAD-containing enzymes

(x-ray crystallography/flavoprotein/nitric-oxide synthase)

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Communicated by Ronald W. Estabrook, University of Texas Southwestern Medical Center, Dallas, TX, May 30, 1997 (received for review May 7, 1997)

ABSTRACT Microsomal NADPH–cytochrome P450 reductase (CPR) is one of only two mammalian enzymes known to contain both FAD and FMN, the other being nitric-oxide synthase. CPR is a membrane-bound protein and catalyzes electron transfer from NADPH to all known microsomal cytochromes P450. The structure of rat liver CPR, expressed in *Escherichia coli* and solubilized by limited trypsinolysis, has been determined by x-ray crystallography at 2.6 Å resolution. The molecule is composed of four structural domains: (from the N- to C- termini) the FMN-binding domain, the connecting domain, and the FAD- and NADPH-binding domains. The FMN-binding domain is similar to the structure of flavodoxin, whereas the two C-terminal dinucleotide-binding domains are similar to those of ferredoxin–$NADP^+$ reductase (FNR). The connecting domain, situated between the FMN-binding and FNR-like domains, is responsible for the relative orientation of the other domains, ensuring the proper alignment of the two flavins necessary for efficient electron transfer. The two flavin isoalloxazine rings are juxtaposed, with the closest distance between them being about 4 Å. The bowl-shaped surface near the FMN-binding site is likely the docking site of cytochrome *c* and the physiological redox partners, including cytochromes P450 and *b5* and heme oxygenase.

Cytochrome P450-mediated microsomal electron transport is responsible for oxidative metabolism of both endogenous compounds, including fatty acids, steroids, and prostaglandins, and exogenous compounds ranging from therapeutic drugs and environmental toxicants to carcinogens. It is mediated by a multicomponent monooxygenase system, in which reducing equivalents from NADPH ultimately are transferred to molecular oxygen (for review, see refs. 1 and 2). In its simplest form, the monooxygenase system consists of NADPH-cytochrome P450 reductase (CPR; NADPH–ferrihemoprotein reductase, EC 1.6.2.4) and one of many cytochrome P450 isozymes (3, 4). Both CPR and microsomal cytochromes P450 are integral membrane proteins, and CPR is one of only two known mammalian enzymes containing both FMN and FAD as prosthetic groups, the other being various isoforms of nitric-oxide synthase (NOS). Other physiological electron acceptors of CPR include microsomal heme oxygenase (5) and cytochrome *b5* (6) and, although nonphysiological, CPR is capable of transferring reducing equivalents to cytochrome *c* (7). By virtue of the fact that only two protein components are required to catalyze the hydroxylation of a number of substrates, this system represents a simple model for other, more complex electron transport systems.

CPR accepts a pair of electrons from NADPH as a hydride ion, with FAD and FMN being the port of entry and exit, respectively, and transfers these electrons one at a time to cytochromes P450. Cytochromes P450, in turn, use these reducing equivalents for the hydroxylation of a variety of substrates. The redox potentials of each flavin half-reaction in the native enzyme have been determined by potentiometric titrations (8, 9). The enzyme cycles between $1e^-$ and $3e^-$ reduced levels (or $2e^-$ and $4e^-$), with the one-electron-reduced semiquinone of the FMN being the highest oxidation state during catalytic turnover (10, 11).

Amino acid sequences for several CPRs from different species ranging from yeast to trout to human reveal high sequence homology, indicating the importance of the enzyme throughout the course of evolution (1). The enzyme has two functional domains, a hydrophobic N-terminal membrane-binding domain and a hydrophilic C-terminal catalytic domain that is comprised of several structural domains. The hydrophobic N-terminal domain (6 kDa) serves to anchor the protein molecule to the endoplasmic reticulum and nuclear envelope (12), thus ensuring proper spatial interaction for electron transfer between the reductase and cytochromes P450. The membrane-bound CPR can be solubilized by limited proteolysis with pancreatic steapsin or trypsin, releasing the C-terminal hydrophilic 72-kDa domain. Without the hydrophobic anchor, the larger soluble domain is still capable of passing electrons to cytochrome *c* and other artificial electron acceptors, but is incapable of transferring electrons to cytochromes P450. Amino acid sequence analysis predicted that this anchor-less C-terminal hydrophilic domain can be further divided into two structural domains. The N-terminal domain, consisting of approximately 170 amino acid residues, is homologous to that of the bacterial flavodoxin, suggesting that this portion of the reductase is likely to bind FMN, and the C-terminal region contains segments with considerable sequence similarity to two NAD(P)H dependent FAD-containing enzymes, ferredoxin–$NADP^+$ reductase (FNR), and NADH–cytochrome *b5* reductase, indicating that this portion binds FAD and NADPH (13). This distinct domain arrangement is consistent with results from further studies in which each domain was expressed independently (14), and unfolding of the polypeptide was correlated with the release of each individual flavin (15). CPR has been studied extensively by a number of biophysical methods, including EPR spectroscopy (16, 17), time-resolved fluorescence spectroscopy (18, 19), ^{31}P NMR (20, 21), and resonance Raman spectroscopy

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Abbreviations: CPR, NADPH–cytochrome P450 reductase; FNR, ferredoxin-$NADP^+$ reductase; NOS, nitric-oxide synthase.

Data deposition: The atomic coordinates have been deposited in the Brookhaven Protein Data Bank, Upton, NY 11973 (ID code, 1amo).

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Table 1. Data collection statistics

Data set	Native	K_2OsCl_6	$PtCl_4$	K_2OsCl_6 + K_2PtCl_6	Thimerosal
Resolution, Å*	2.6 (2.76-2.6)	3.0 (3.25-3.0)	3.0 (3.25-3.0)	3.0 (3.23-3.0)	2.8 (3.23-3.0)
Unique reflections	41,132	26,064	27,320	20,104	26,599
Total observations	171,499	128,475	152,158	120,324	113,336
Completeness, %	91.5 (85.8)	87.6 (78.8)	91.7 (85.7)	68.1 (42.0)	89.1 (85.5)
R_{sym}, %†	7.0 (30.8)	6.4 (20.4)	6.2 (17.3)	5.8 (22.7)	8.6 (25.2)
R_{iso}, %‡	—	13.5	12.4	15.3	17.0
Soaking conditions	—	1 mM/36 hr	1 mM/18 hr	1 mM/4 hr + 1 mM/20 hr	3 mM/72 hr
Number of sites	—	2	4	6	4
Phasing power, 3 Å§	—	1.67 [0.80]	1.09 [0.93]	1.32 [0.60]	1.14 [0.83]
R_{cullis}	—	0.666	0.670	0.676	0.689
Mean figure of merit	0.596	0.352 [0.194]	0.222 [0.218]	0.274 [0.123]	0.237 [0.193]

The final crystallographic *R*-factor was 20.0% with *R*-free of 31.0% for a total of 10,156 nonhydrogen atoms, including 78 solvent atoms.
*Highest resolution shell statistics are given in parenthesis; statistics for anomalous data sets are shown in brackets.
†$R_{sym} = \Sigma_h\Sigma_i|I_{hi} - \langle I_h\rangle|/\Sigma_h\Sigma_i I_{hi}|$, where h are unique reflection indices and i indicate symmetry equivalent indices.
‡$R_{iso} = \Sigma||F_{ph}| - |F_p||/\Sigma|F_p|$ for isomorphous replacement data.
§Phasing power = f_{rms}/E_{rms}, where $f_{rms} = [(\Sigma f_h^2)/n]^{1/2}$ and $E_{rms} = [\Sigma(F_{ph} - |F_p + f_h|)^2/n]^{1/2}$.

(22). Despite these intensive studies, little has been determined about physical and electronic interactions between FMN, FAD, and NADPH and interactions between CPR and its electron transfer partners. We now describe the complete three-dimensional structure of rat liver CPR expressed in *Escherichia coli* and treated by limited trypsinolysis. The structure represents a prototype for the spatial arrangements of the two flavins in the enzyme and provides insights into not only the interactions of CPR with its physiological electron acceptors, including cytochromes P450, but also the mechanism of electron-transfer and its regulation in other FMN- and FAD-containing enzymes, including NOSs.

Purification and Crystallization of CPR. Rat liver CPR was cloned in *E. coli* and purified as described (23). Purification of the full-length CPR protein involved centrifugation, solubilization using Triton X-100, ultracentrifugation, and finally affinity chromatography on a 2′,5′-ADP Sepharose 4B affinity column in the presence of Triton X-100. The holo CPR was treated with immobilized trypsin, and the clipped CPR (hereafter referred to simply as CPR) was further purified in the absence of any detergent. Final purification of the clipped enzyme required hydroxyapatite chromatography using a Bio-Rad ceramic FPLC column.

The purified CPR was crystallized as previously described (24). Crystallization of CPR required adding excess FMN and $NADP^+$ during the concentration/dilution procedure, and any excess cofactors were removed before setting up the crystallization trials. Crystals were obtained by the vapor diffusion method; the final crystallization involved mixing equal volumes of CPR (25 mg/ml) and precipitant (21% PEG 4500/150 mM Hepes, pH 6.5/5 mM $MgCl_2$/0.8 M NaCl), and equilibrating against precipitant at 19°C. Crystals routinely grew to approximately 0.3 mm × 0.5 mm × 0.6 mm within 2–3 weeks. Data collection was performed on an R-AXIS II image plate system at 4°C. The CPR crystals belong to the orthorhombic space group $P2_12_12_1$, with unit cell parameters $a = 103.28$ Å, $b = 116.18$ Å, and $c = 119.77$ Å. Assuming two molecules per asymmetric unit, the calculated V_m value is 2.49 Å³/Da (25).

Structure Determination of CPR. The structure was solved using multiple isomorphous replacement methods together with anomalous scattering data. Self-rotation function analysis was not successful, but self-Patterson analysis revealed a large peak around (u, v, w)= (1.0, 0.22, 0.5), indicating that the local 2-fold axis is parallel to the *z*-axis, located near $(x, y) = (0.25, 0.11)$. During heavy atom screening, the degree of isomorphism between the native data set and the derivative data sets was monitored by following the position of the self-Patterson peak. If the peak position varied by more than 3%, it was not considered isomorphous to the native data and was not used for phasing analysis. This nonisomorphism was not necessarily a result of the derivative soaking, because oftentimes two different native crystals showed the same trend in nonisomorphism. Four isomorphous derivatives were obtained that were suitable for obtaining the initial phases (Table 1). The positions and occupancies of the heavy atoms were initially refined using the program HEAVY (26), and multiple isomorphous replacement phases were calculated and refined using the PHASES package (27). Initial electron density maps were calculated using data between 30 and 3.0 Å resolution. The phases were further improved by density modification

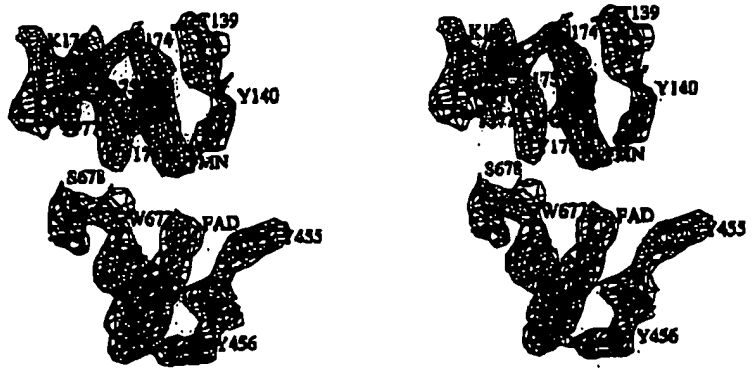

FIG. 1. Stereoview of the electron density in the vicinity of the FAD and FMN of CPR. The $3|F_o| - 2|F_c|$ electron density map computed with 2.6 Å resolution data is contoured at 1.2σ level. Residues within close contact of the flavin ring are labeled. Four aromatic residues sandwich the flavin rings: Y178 and Y140 for FMN and Y456 and W677 for FAD.

using the solvent flattening procedure, as well as noncrystallographic symmetry averaging (28). At this time, well defined α-helices and β-strands were easily identified. A polyalanine model was then constructed using the molecular graphics program TURBO-FRODO (29) on a silicon graphics workstation.

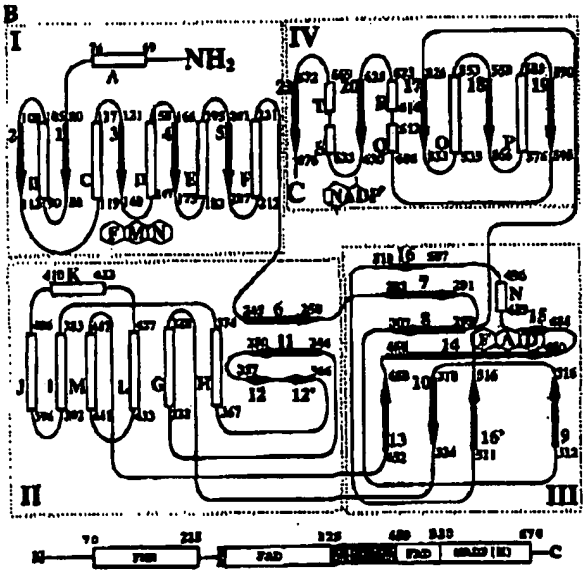

FIG. 2. Overall polypeptide fold and topology diagram for CPR. (*A*) Ribbon diagram showing the structure of CPR. The FMN-binding domain is shown in blue, the connecting domain in red, and the FAD- and NADP(H)-domains are represented in green. The cofactors are shown as ball and sticks, with the FMN (light blue), FAD (yellow), and NADP+ (orange). A disordered region that represents a "hinge" is shown in pink (between the FMN-binding and the connecting domain). The drawing was generated using MOLSCRIPT (32) and RASTER3D (33). (*B*) Topology diagram of the CPR protein, indicating regions responsible for cofactor recognition. α-Helices (open cylinders), β-strands (filled arrows), random coils (lines), and corresponding residue numbers are indicated. The FMN domain (I), connecting domain (II), FAD domain (III), and NADP+ domain (IV) are identified by boxes. Domains II and III are not contiguous in linear sequence, and the last two nucleotide binding domains (III and IV) form an FNR-like structure. The α-helices are lettered, and the β-strands are numbered sequentially from N to C termini. A linear diagram showing domain arrangement in the CPR structure is shown at the bottom. Each cofactor binding domain is indicated, and the connecting domain is depicted as stippled boxes. The numbers above the boxes indicate approximate amino acid positions for the corresponding domains in the CPR structure. The connecting domain and the FAD-binding domain are intertwined in linear sequence, yet both of them make distinctive structural domains.

The location of the FAD and FMN cofactors was easily identified (Fig. 1), and the structures of flavodoxin and FNR were superimposed onto the CPR density, aiding in chain-tracing. After building the initial model of CPR, electron density maps were generated by combining the multiple isomorphous replacement phases and those of the model according to the SIGMAA algorithm (30). Phase combination allowed for completion of the chain tracing. After several cycles of manually adjusting the model to fit the electron density maps, along with additional sequence assignment followed by refinement using the X-PLOR package (31), the entire sequence was assigned. The polypeptide connectivity was confirmed by calculating simulated annealing omit maps. The first seven residues (residues 57 to 63) at the N terminus and 10 residues in the hinge region (residues 234 to 243) are disordered in the refined structure. The final *R*-factor was 20.0%, with an R_{free} of 31.0%, including 78 water molecules, for 37,048 reflections between 10 and 2.6 Å resolution with $I/\sigma > 2.0$. The rms deviations of the bond lengths and bond angles of the final structure are 0.007 Å and 1.5°, respectively.

The Overall Structure and Domain Organization. Sequence analysis of dissolved crystalline protein revealed that the N terminus residue is Ile-57. The structure, however, as seen in the crystallographic electron density map, begins with Val-64 indicating that the N terminus of the hydrophilic domain of CPR is flexible. This is consistent with biochemical studies indicating that this region is highly exposed in the intact enzyme and is very susceptible to proteolytic cleavage, as well as crystallographic studies in which we failed to crystallize the intact CPR protein, due to persistent cleavage at this region. An overview of the backbone structure of CPR in ribbon representation and its topology diagram are shown in Fig. 2. The structure of CPR is composed of four structural domains. From N to C terminus, they are the FMN-binding domain, the connecting domain, and the FAD- and the NADP-binding domains, consistent with the previous prediction from the sequence alignment (13). Similar to the flavodoxin structure from *Desulfovibrio vulgaris* (34), the FMN binding domain consists of a five-stranded parallel β-sheet flanked by five α-helices (Fig. 2*B*) with the FMN positioned at the tip of the C-terminal side of the β-sheet. As seen in the structures of FNR (35) and other FNR-like enzymes such as cytochrome *b5* reductase (36) and corn nitrate reductase (37), the core of the FAD-binding domain is an anti-parallel flattened β-barrel, and that of the NADP(H)-binding domain is another parallel five-stranded β-sheet sandwiched by α-helices. The connecting domain, situated between the two flavin domains, consists of residues between 240 to 270 and between 325 to 450 and is comprised mainly of α-helices. This domain appears to be responsible for bringing the two flavins together and, there-

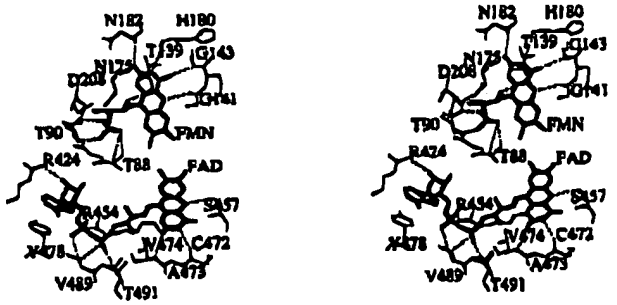

FIG. 3. Stereoview showing residues in the vicinity of the FAD and FMN cofactors. The FAD and FMN cofactors in CPR are oriented such that the xylene portions of both isoalloxazine rings are pointed toward each other. The FAD is in an extended conformation. Potential hydrogen bonds are indicated by dotted lines. For clarity, the aromatic residues sandwiching both isoalloxazine rings shown in Fig. 1 are not drawn in this figure.

fore, for modulating electron transfer between the two flavins. The loop between helix F and strand 6 is not well ordered in the crystal structure (Fig. 2*A*) and forms a hinge between the FMN-binding domain and the connecting domain (Fig. 2*B*). Residues located at the interface between the FMN binding- and the connecting-domains are mainly hydrophilic, and, therefore, the interaction between them is largely electrostatic. The location of FMN in the FMN-binding domain and movement of this domain that exposes the FMN to solvent are consistent with previous biochemical findings that describe the ease with which FMN dissociates from the enzyme (9, 15), as movement of this domain would coincide with FMN release/binding. On the other hand, the interfaces between the connecting domain and the FAD-binding domain, and between the FAD- and the NADP(H)-binding domains are relatively hydrophobic, again explaining the sequential release of FAD only after protein unfolding upon treatment with chaotropes. Furthermore, the linear sequence for the latter three domains are intertwined, and therefore these domains may not be functionally separable. The overall shape of the molecule as viewed in Fig. 2*A* is an oval-shaped bowl that is approximately 50 Å deep × 70 Å wide × 60 Å high, and the cofactors lie in the middle of the bowl.

FMN- and FAD-Binding Sites. FMN is located at the C-terminal side of the β-strands in the FMN-binding domain (Fig. 2*A*). As seen in Figs. 1 and 3, the two flavins do not overlie each other, but rather are end on and communicate with each other through the 7- and 8-methyl groups of the xylene rings. The two isoalloxazine rings make an angle of about 150° to each other, forming an almost continuous "ribbon" with the closest distance between them being 3.5 Å between the C_7M atoms and 4.5 Å between the C_8M atoms of the xylene rings. This arrangement indicates that the electron flow between the two flavins is not mediated by any amino acid residue atoms, and instead is direct, consistent with kinetic data indicating that the electron transfer between the two flavins is rapid. Both ends of the flavin ribbon are made up of the pyrimidine side of the isoalloxazine ring and are hydrogen bonded to the main chain atoms of the polypeptide (Fig. 3). FMN lies at the C-terminal tip of the β-sheet in the FMN-binding domain and is bound to polypeptide fragments ^{139}TYGEGPD and ^{175}NKTYEHFN, and the FAD is encompassed by fragments ^{455}YYSIAS and ^{471}ICAVAV. The isoalloxazine ring of FMN is covered by the phenolic ring of Y140 at the re-side and Y178 at the si-side (Fig. 1). As observed in the FNR structure (35), the FAD is bound in an extended conformation, and the isoalloxazine ring is inserted at the boundary between the FAD- and the NADP(H)-binding domains, whereas the rest of the FAD molecule lies at the interface between the FAD-binding domain and the connecting domain. The re-side of the FAD ring is stacked with the indole ring of W677 and Y456 lies at its si-side (Fig. 1). As in the FNR structure, the pyrophosphate is stabilized by side chains from T491 and R454, and the polypeptide segment of residues C472 to Y478 is almost parallel to the ribityl-pyrophosphate chain. The adenosine moiety is rather exposed, and the phenolic ring of Y478 is stacking on one side of the adenine ring (Fig. 3).

$NADP^+$ Adopts Multiple Conformations. The electron density for the ribose-nicotinamide moiety of the bound $NADP^+$ is only about 30% of that of the rest of the molecule in both molecules of the asymmetric unit. Furthermore, its conformation is different in the two structures, indicating that this portion of the molecule is highly flexible and disordered. Although the exact conformation of the ribose-nicotinamide moiety cannot be defined from current data, an approximate model can be built in each structure, only to be used to estimate the relative orientation of the bound $NADP^+$ with respect to the FMN and FAD (Fig. 4). The two observed conformations are easily interconvertible by changing the dihedral angle by about 120° of the nicotinamide side of the pyrophosphate ($—P_N—O—P_A—$) bond. However, in both conformations, the distance between the C4 atom of the nicotinamide (the hydride donor) and the N5 of the FAD isoalloxazine ring (the hydride acceptor) is over 9 Å in one and over 14 Å in the other conformer, indicating that neither of these conformations of $NADP^+$ is capable of direct hydride transfer to FAD. Therefore, the enzyme-$NADP^+$ complex structure observed in both crystal forms must represent the enzyme-product complex of the reductive half reaction. It should be noted that, by changing the dihedral angles of both sides of the bridging pyrophosphate oxygen atom ($—P_N—O—P_A—$) of the $NADP^+$ molecule, the nicotinamide ring could be positioned at the re-side of the FAD ring where W677 lies (Fig. 4), in a manner similar to that observed in the glutathione reductase structure and to that suggested by Karplus *et al.* (35) for the FNR structure. In the case of glutathione reductase, the phenolic ring of Y197 covers the si-face of the FAD ring in the oxidized state, but flips away to make room for the nicotinamide ring of the NADPH when it binds to the enzyme to form a reduced enzyme-NADPH complex (38). Despite numerous attempts, no crystal structure is available of an FNR family member showing nicotinamide nucleoside conformation in an enzyme-NAD(P)(H) complex. No electron density was observed for the nicotinamide nucleoside moiety in the crystal structure of the reduced FNR-NADPH complex (39). Interestingly, similar results were observed in the studies of phthalate dioxygenase reductase (40), a member of an extended FNR family in which a

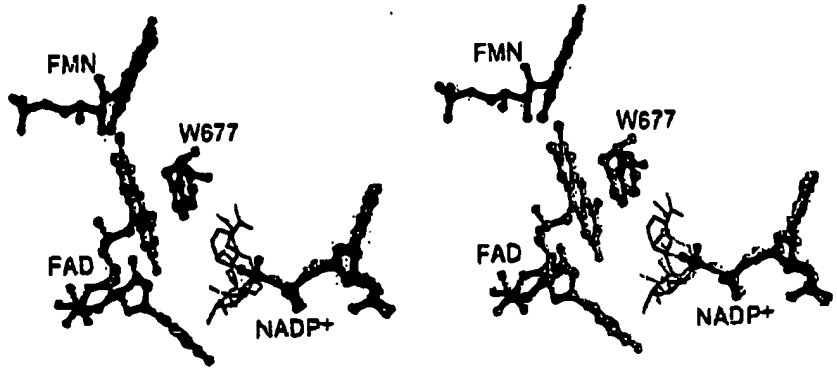

FIG. 4. Stereoview of the cofactor arrangement in CPR and multiple conformers for the bound $NADP^+$. Electrons flow from NADPH to FAD and then to FMN. The FMN and FAD are represented by ball-and-stick, with the xylene portions of the isoalloxazine rings oriented toward each other. The adenine portion of $NADP^+$ binds in a single conformation (ball-and-stick) while the nicotinamide (stick only) binds in multiple conformations. By rotation about the $P_N—O—P_A$ bond, the nicotinamide ring could displace W677 at the re-side of the FAD ring, placing it in the optimum orientation for hydride transfer from the NADPH to the N5 position of the FAD cofactor.

ferredoxin-like [2Fe–2S] domain is attached to the C terminus of an FNR-like module.

Interactions with Cytochrome *c* and Cytochromes P450. As the soluble form of CPR is capable of transferring electrons from NADPH to cytochrome *c*, we attempted to map out the cytochrome *c* binding surface of the CPR molecule. Because the pyrimidine side of the FMN isoalloxazine ring lies close to the solvent accessible surface of the CPR molecule, it is reasonable to assume that electrons must exit from this side of the FMN through the FMN-binding domain and enter the heme of the acceptor molecule. Thus, the FMN-binding domain must provide a major portion of the docking surface. This is further supported by the fact that bacterial flavodoxin can serve to transfer electrons to cytochromes P450, either combined with the NADPH-reduced flavodoxin reductase

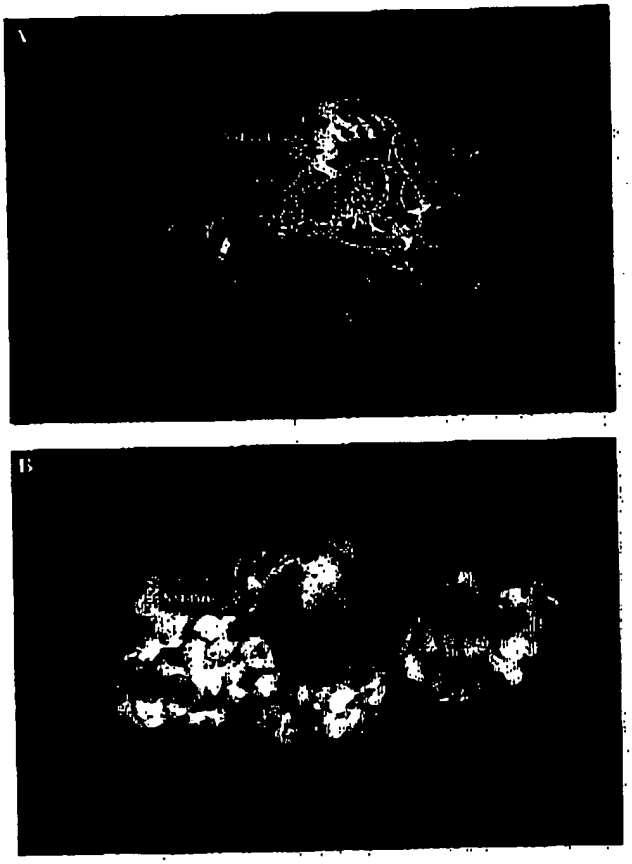

FIG. 5. Putative docking surface of CPR with its electron transfer partners. (*A*) Ribbon diagram showing the putative docking of CPR and cytochrome *c* in an orientation that represents CPR "sitting" on the membrane surface. The N-terminal membrane anchor portion of CPR, which was proteolytically removed for crystallization studies, would protrude downwards, into the membrane. Cytochrome *c* (or cytochrome P450) binds "in front of" the CPR molecule, allowing the NADP(H) to enter/exit freely from above, while the FMN and FAD are buried in the complex. The domains and cofactors of CPR are colored as shown in Fig. 2*A*, and cytochrome *c* is in pink. Because the cytochrome P450 is membrane-bound, interactions between the membrane domains of the two proteins would be possible in this "side-by-side" orientation. (*B*) The electrostatic potential mapped onto the solvent accessible surface of CPR (*Left*) and cytochrome *c* (*Right*). The view for CPR is the same as in *A*. The positive potential is shown in blue, and the negative potential is shown in red. The potential was calculated with Delphi (43) using formal charges and contoured at 2.5 *kT*, and mapped onto the solvent-accessible surface using GRASP (44). From this view, it is clear that the interactions between CPR and cytochrome *c* are primarily electrostatic in nature. The putative surface of CPR onto which cytochrome *c* binds is indicated with a dotted circle.

(41) or when electrochemically reduced using dye (42). Fig. 5*A* shows a plausible mode of docking between cytochrome *c* and CPR that minimizes the distance between the two redox centers (FMN and the heme) and allows a maximum contact area between the two molecules. It also allows a positively charged cytochrome *c* surface to bind to the mostly negatively charged CPR surface. The model was obtained by manual inspection of two structures, followed by rigid body energy minimization using the X-PLOR package. The closest distance between the FMN of CPR and the heme of cytochrome *c* is less than 9 Å. The electrostatic potential mapped onto the docking surfaces of CPR and cytochrome *c* molecules are shown (Fig. 5*B*). The CPR docking surface contains the loop between strand 4 and helix E, consisting of residues 175 to 182, and the loop between strand 5 and helix F, containing residues 207 to 211 (Fig. 2*B*). The latter loop contains the acidic residues that were indicated to be involved in the binding of CPR to cytochrome *c* and cytochromes P450 by chemical crosslinking (45) and site-directed mutagenesis studies (46). This surface is, most likely, a major portion of the cytochromes P450 binding surface as evidenced by the inhibition of cytochromes P450 reactions by cytochrome *c* (47). A large portion of this surface also would overlap with the cytochrome *b5* binding surface. Because cytochromes P450 are larger molecules than cytochromes *c* or *b5*, the contact surface with CPR would be larger, therefore, the CPR "bowl" would be filled to its rim with the P450 molecule. Furthermore, for the membrane-bound electron transfer partners, hydrophobic interactions between the membrane domains of the intact CPR and cytochromes P450 (or cytochrome *b5*) must play a significant role in addition to the ionic interactions described above.

Membrane-Binding Site. The N terminus of the CPR molecule is situated at the opposite side of the $NADP^+$ binding site (back side of the molecule shown in Fig. 2*A*). The membrane-binding site of the CPR structure is likely to be situated around Val-64 and near some hydrophobic patches of the CPR surface, most likely, containing loops 516–525, 250–281, and 553–557. Fig. 5*A* illustrates the orientation of a CPR molecule "sitting" on the membrane surface. The NADP(H) molecule is bound on the cytoplasmic side of the molecule, and the N terminus is facing to the membrane side. In this view, cytochrome *c* or other electron transfer partners would bind "in front of" the molecule where it can communicate with the FMN most directly. This "side-by-side" orientation of P450 and CPR would make it simpler for the two membrane-bound molecules to interact on the membrane.

The CPR Family. CPR belongs to a family of enzymes that contain both FMN and FAD and shuttles electrons between pyridine nucleotide and heme via FAD and FMN. In addition to CPR, the family includes P450BM-3, NOS, and bacterial sulfite reductase. It is a subfamily of the FNR superfamily, a structurally designated family of proteins whose common feature is an FNR-like domain (48). Amino acid sequence analysis of the CPR family members reveals striking similarities among them, ranging from 50% between CPR and the α-subunit of sulfite reductase to 58% between CPR and the C-terminal 640 residues of neuronal NOS (49). A sequence alignment for the cofactor-binding regions of the flavin domains of P450-BM3, rat neuronal NOS, and the α-subunit of *E. coli* sulfite reductase, along with CPR, is shown in Fig. 6. In addition, using structural alignment, the N-terminal FMN-binding domain was aligned with flavodoxin, whereas the C-terminal portion was aligned with the FNR structure. Sequences for most of the secondary structural elements are conserved, as well as all cofactor binding sites, indicating that the polypeptide folding and the cofactor arrangement in the structures of other members of the CPR family are very similar to those of CPR. Thus, the structure of CPR can serve as a model for understanding the mechanism and function of the other members of the CPR family. Furthermore, it provides a

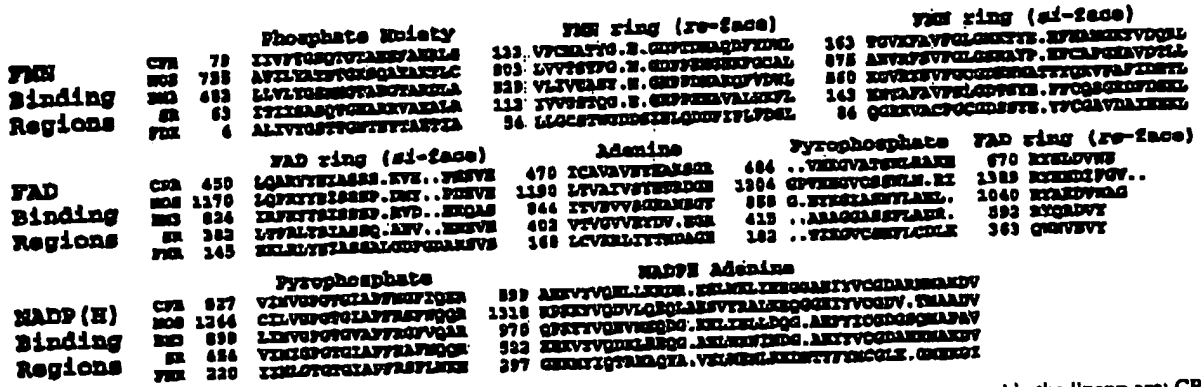

FIG. 6. Sequence lineup for cofactor-binding regions of key members of the CPR family. The proteins represented in the lineup are: CPR, rat cytochrome P450 reductase; NOS, rat neuronal nitric-oxide synthase; BM3, cytochrome P450 BM-3; SR, *E. coli* sulfite reductase; FDX, *D. vulgaris* flavodoxin; and FNR, *Spinacia oleracea* FNR. The numbering is for the first amino acid of each region for the given protein. The lineups of FDX and FNR were made by superimposing the molecular structures onto the CPR structure.

model for studying not only flavin–flavin interactions, but also electron transfer mechanisms among flavoproteins, in general.

We thank J. P. Fulmer for making Fig. 5*B*. This work was supported by Grants GM52682 (J.-J.P.K.) and HL30050 (B.S.S.M.) from the National Institutes of Health.

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